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(54) Title: CONTROLLING NK-1 RECEPTOR-MEDIATED RESPONSES AND RELATED DIAGNOSTICS (57) Abstract cDNA encoding the human NK-1 receptor is cloned and the recombinant protein expressed. Recombinant receptor and receptor fragments are used as therapeutics to reduce pain and treat disorders, such as inflammatory diseases and mental health disorders. The recombinant receptor and receptor fragments are also used in methods of screening candidate compounds for the ability to antagonize interaction between the substance P neurotransmitter and NK-1 receptor. Antibodies specific for NK-1 receptor and their use as a therapeutic and/or a vaccine is also disclosed.		

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**CONTROLLING NK-1 RECEPTOR-MEDIATED RESPONSES
AND RELATED DIAGNOSTICS**

BACKGROUND OF THE INVENTION

5 This invention relates to receptors, particularly receptors involved in neurotransmission.

 Substance P (SP) is one member of a family of peptide neurotransmitters known as tachykinins (von Euler et al., *J. Physiol.* 72:74, 1931; Chang et al., *J. Biol.* 10 *Chem.* 245:4784, 1970).

 Molecular characterization has revealed that tachykinins are transcribed from alternatively-spliced mRNA molecules (termed, α , β and γ mRNAs) and are translated as precursor molecules, termed 15 preprotachykinins (Nawa et al., *Nature* 306:32, 1983; Kawaguchi et al., *Biochem. Biophys. Res. Commun.* 139:1040, 1986; Krause et al., *Proc. Natl. Acad. Sci, USA* 84:881, 1987). Specifically, the β and γ messages encode preprotachykinins which include substance P and another 20 tachykinin, neurokinin A; the β mRNA molecule encodes an amino-terminally extended form of neurokinin A, termed neuropeptide K or NpK; and the α form encodes only substance P. Mature tachykinin molecules are produced from the preprotachykinins by proteolytic processing. 25 Structurally, the tachykinin family shares the COOH-terminal protein sequence: Phe-X-Gly-Leu-Met-NH₂ (SEQ ID NO.:1), where X is Phe, Tyr, Val, or Ile. Substance P, in particular, is of amino acid sequence: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂ (SEQ ID NO.:2).

30 Three classes of tachykinin receptors have been identified by bioassay and radioligand binding (Martling et al., *Life Sci.* 40:1633, 1987; Buck et al., *Science* 226:987, 1984; Burcher et al., *J. Pharmacol. Exp. Ther.* 236:819, 1986). Analysis of these receptors has revealed 35 a COOH-terminal consensus sequence of the receptors which

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controls biological activity and divergent amino-terminal sequences which determine receptor affinity. The result of such an arrangement is that each tachykinin recognizes each of the three receptor types, but with varying
5 avidity. In particular, the NK-1 receptor preferentially binds substance P; the NK-2 receptor preferentially binds neurokinin A; and the NK-3 receptor preferentially recognizes neurokinin B (another tachykinin, remote from neurokinin A or substance P). Synthetic tachykinin
10 analogs designed to act as competitive inhibitors exhibit relative selectivity for each of the three neurokinin receptors (Wormser et al., *EMBO J.* 5:2805, 1986; Cavinikas et al., *Eur. J. Pharmacol.* 77:205, 1982; Regoli et al., *Tachykinin Antagonists*, eds. R. Hakanson and F.
15 Sundler) pp. 277-287, Elsevier Science Publisher B. V., Amsterdam, 1985).

Tachykinins, in general, have been found to display a wide tissue distribution and to be involved in numerous physiological activities (Lee et al., *Eur. J. Pharmacol.* 130:209, 1986; Lundberg et al., *Acta Physiol. Scand.* 119:243, 1983; Nicoll et al., *Ann. Rev. Neurosci.* 3:227, 1980; Pernow, *Pharmacol. Rev.* 35:85, 1983; Maggio, *Ann. Rev. Neurosci.* 11:13, 1988). Such activities include vasodilation (Pernow and Rosell, *Acta. Physiol. Scand.* 93:139, 1975), smooth muscle contraction (Pernow, *Acta Physiol. Scand.* 93 Suppl. 105:1, 1953), stimulation of salivary secretion (Vogler et al., *N.Y. Acad. Sci.* 104:378, 1963; Leeman and Hammerschlag, *Endocrinology* 81:803, 1967), transmission of painful stimuli from the
30 peripheral nervous system, and interactions with other neurotransmitters in the brain (Nicoll et al., *Ann. Rev. Neurosci.* 3:227, 1980; Maggio, *Ann. Rev. Neurosci.* 11:13, 1988; Nilsson et al., *Med. Biol.* 52:424, 1974; Hokfelt et al., *Science* 190:889, 1975; Mantyh et al., *Nature*
35 309:795, 1984; Womack et al., *Nature* 334:351, 1988).

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Substance P has also been found to play a role in the activation of cells of the immune system, including mononuclear leukocytes (e.g., monocytes and lymphocytes) and polymorphonuclear leukocytes (Payan et al., *J. Clin. Invest.* 74:1532, 1984; Hartung et al., *J. Immunol.* 136:3856, 1986; Lotz et al., *Science* 235:893, 1987; Perianin et al., *Biochem. Biophys. Res. Commun.* 161:520, 1989).

SUMMARY OF THE INVENTION

10 In one aspect, the invention generally features human recombinant NK-1 receptor or a fragment thereof. Preferably, the receptor includes an amino acid sequence substantially identical to the amino acid sequence shown in Fig. 1 (SEQ ID NO.:3). By a "substantially identical"

15 amino acid sequence is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g., valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino

20 acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the biological activity of the receptor. Such equivalent growth factors can be isolated by extraction from the tissues or cells of any animal which naturally produce

25 such a receptor or which can be induced to do so, using the methods described below, or their equivalent; or can be isolated by chemical synthesis; or can be isolated by standard techniques of recombinant DNA technology, e.g., by isolation of cDNA or genomic DNA encoding such a

30 receptor.

In a related aspect, the invention features a substantially isolated polypeptide which is a fragment of a human NK-1 receptor and includes an extracellular domain capable of binding substance P neurotransmitter.

35 By a "polypeptide" is meant any chain of amino acids,

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regardless of length or post-translational modification (e.g., glycosylation). A "substantially isolated polypeptide" is one which is substantially free of other proteins, carbohydrates and lipids with which it is naturally associated. By an "extracellular domain" is meant any portion of the protein (in this case, the human NK-1 receptor) which is exposed on the outer surface of a receptor-bearing cell, and which contains significant structural information to participate in or define specific binding.

In preferred embodiments, the polypeptide is selected from the group including:

- (a) MDNVLPVDSDLSPNISTNTSEPNQFVQPAWQ (SEQ ID NO.:4; amino acid residues 1 to 31 of Fig. 1; SEQ ID NO.:3);
- (b) FTYAVHNEWYYG (SEQ ID NO.:5; amino acid residues 90 to 101 of Fig. 1; SEQ ID NO.:3);
- (c) TTETMPSRVVCMIIEWPEHPNKIYEKVYH (SEQ ID NO.:6; amino acid residues 170 to 197 of Fig. 1; SEQ ID NO.:3);
- (d) PYINPDLYLKKFIQQVY (SEQ ID NO.:7; amino acid residues 271 to 287 of Fig. 1; SEQ ID NO.:3); and (e) fragments or analogues of (a)-(d) which are capable of binding human NK-1 neurotransmitter. Preferably, such a polypeptide is a recombinant polypeptide.

In other related aspects, the invention features purified DNA which encodes a receptor (or fragment thereof) or a polypeptide described above; vectors which contain such DNA and are capable of directing expression of the protein encoded by the DNA in a vector-containing cell; and cells containing such vectors (preferably eukaryotic cells, e.g., mammalian cells). By "purified DNA" is meant a DNA molecule which encodes the human NK-1 receptor (or an appropriate receptor or analog), but which is free of the genes that, in the naturally-occurring genome of the organism from which the DNA of

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the invention is derived, flank the gene encoding the NK-1 receptor.

The expression vectors or vector-containing cells of the invention can be used in a method of the invention to produce human NK-1 receptor and the polypeptides described above. The method involves providing a cell transformed with DNA encoding the NK-1 receptor or a fragment thereof positioned for expression in the cell; culturing the transformed cell under conditions for expressing the DNA; and isolating the recombinant human NK-1 receptor protein. By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of genetic engineering, a DNA molecule encoding the human NK-1 receptor (or a fragment or analog, thereof). Such a DNA molecule is "positioned for expression" meaning that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (i.e., facilitates the production of the NK-1 receptor protein, or fragment or analog, thereof).

In yet another aspect, the invention features purified antibody which binds preferentially to the human NK-1 receptor (or a fragment thereof) or a polypeptide described above. By "purified antibody" is meant one which is sufficiently free of other proteins, carbohydrates, and lipids with which it is naturally associated to permit therapeutic administration. Such an antibody "preferentially binds" to a human NK-1 receptor (or fragment or analog, thereof), i.e., does not substantially recognize and bind to other antigenically-unrelated molecules. Preferably, the antibody neutralizes the biological activity in vivo of the protein to which it binds. By "biological activity" is meant the ability of the NK-1 receptor to bind substance P and signal the appropriate cascade of biological

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events. By "neutralize" is meant to partially or completely block (e.g., the biological activity of the human NK-1 receptor).

In other aspects, the receptor, polypeptides, or antibodies described above are used as the active ingredient of therapeutic compositions. In such therapeutic compositions, the active ingredient may be formulated with a physiologically-acceptable carrier or anchored within the membrane of a cell. These therapeutic compositions are used in methods of reducing pain, treating inflammatory diseases (e.g., arthritis or asthma), treating mental illnesses (e.g., schizophrenia), and treating physiological responses to stress. ("Stress" as used herein means the reaction of the body to forces of a deleterious nature, infections, and various abnormal states that tend to disturb homeostasis). The methods involve administering to a mammal the therapeutic composition in a dosage effective to antagonize an interaction between substance P neurotransmitter and an NK-1 receptor.

Finally, the invention features a method of screening candidate compounds for their ability to antagonize interaction between substance P neurotransmitter and an NK-1 receptor. The method involves: a) mixing a candidate antagonist compound with a first compound which includes a recombinant NK-1 receptor (or fragment) or a polypeptide or an antibody described above on the one hand and with a second compound which includes substance P on the other hand; b) determining whether the first and second compounds bind; and c) identifying antagonistic compounds as those which interfere with the binding of the first compound to the second compound. By an "antagonist" is meant a molecule which inhibits a particular activity, in this case, the ability of substance P to interact with the NK-

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1 receptor and/or to trigger the biological events resulting from such an interaction.

The proteins of the instant invention are involved in a wide range of physiological activities and therefore are useful to treat or, alternatively, to develop therapeutics to treat a variety of disorders (e.g., molecules which antagonize a substance P: NK-1 receptor interaction). The disorders to be treated include pain, particularly chronic pain; diseases arising from inappropriate inflammatory responses, such as arthritis and asthma; and mental-health disorders, such as schizophrenia, anxiety, stress, and the like. Preferred therapeutics include antagonists, e.g., peptide fragments, antibodies, or drugs, which block substance P or NK-1 receptor function by interfering with the neurotransmitter:receptor interaction. Because the receptor component may now be produced by recombinant techniques and because candidate antagonists may be screened in vitro, the instant invention provides a simple and rapid approach to the identification of useful therapeutics. Once identified, a peptide- or antibody-based therapeutic may also be produced, in large quantity and inexpensively, using recombinant and molecular biological techniques.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

DETAILED DESCRIPTION

The drawings will first briefly be described.

DRAWINGS

FIG. 1 shows the complete nucleic acid and deduced amino acid sequence of a cDNA encoding the human NK-1 receptor gene (i.e., SEQ ID NO.:3).

CLONING OF THE HUMAN NK-1 RECEPTOR GENE

The human NK-1 receptor was cloned as follows.

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Human IM-9 lymphocyte cells (ATCC Accession No. CCL 159) were cultured by standard techniques. RNA was extracted from approximately 10^8 cells by first freezing in liquid nitrogen and then extracting with guanidium thiocyanate (by the methods of Ullrich et al., *Science* 196:1313, 1977; and Chirgwin et al., *Biochemistry* 24:5294, 1979), and centrifuging through cesium chloride by the method of Gilsin et al. (*Biochemistry* 13:2633, 1974). Poly(A)⁺ RNA was then isolated by passing the material over oligo(dT)-Sepharose (Pharmacia, Piscataway, NJ) by the method of Aviv and Leder (*Proc. Natl. Acad. Sci. USA* 69:14087, 1972) and transcribed into cDNA by the method of Gubler and Hoffman (*Gene (Amst.)* 25:263, 1983).

Synthetic oligonucleotide primers were designed based on the cDNA sequence reported for the rat NK-1 receptor (Nakanishi et al., *J. Biol. Chem.* 264:17649, 1989). These primers extended from nucleotide 85 to 105 (i.e., the sense primer) and from nucleotide 538-558 (i.e., the antisense primer); each included *EcoRI* restriction site sequences at their 5' ends. Such primers were, respectively, of sequence:

5'-AATGAATTCACCTGGCAAATCGTTCTTTGG-3' (SEQ ID NO.:8)

5'-AATGAATTCCTCCGGCCACTCGATCATGCA-3' (SEQ ID NO.:9)

The primers were synthesized by standard cyanoethyl phosphoramidite chemistry using an Applied Biosystems Model 318A DNA Synthesizer (Foster City, CA).

Approximately 100 ng of human IM-9 lymphocyte cell cDNA was combined with 1 μ g of each of the synthetic primers and recombinant Taq DNA polymerase (Perkin-Elmer, Norwalk, CT). Twenty-five cycles of polymerase chain reaction were carried out by the method of Gerard et al. (*J. Biol. Chem.* 265:20455, 1990). Each cycle included: 1 min at 95°C, 2 min at 37°C, and 2 min at 72°C. This was followed by a final extension period of 7 min at 72°C. The PCR product, an approximately 500 base pair

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fragment including nucleotides 85 to 555, was purified, following electrophoretic separation, using GeneClean (Bio 101, La Jolla, CA). The fragment was digested with *EcoRI*, repurified as above following a second round of electrophoresis, and ligated to *EcoRI*-digested pBluescript SK+ (Stratagene, La Jolla, CA). Clones were amplified in *Escherichia coli* XL-1blue (Stratagene, La Jolla, CA), purified by centrifugation through cesium chloride (by the method of Birnboim and Doly, *Nucl. Acids Res.* 7:1513, 1979), and subjected to double-stranded nucleic acid sequencing using DNA polymerase and [³⁵S]dATP by the method of the supplier (Sequenase, United States Biochemical Corporation, Cleveland, OH). The fragment exhibited 90% sequence identity with the rat molecule.

15 A human placental genomic DNA library, prepared by standard techniques, was screened by Southern blot analysis using the 500-bp NK-1 receptor fragment (isolated above) labelled with ³²P. Bacteriophage DNA was transferred to nitrocellulose filters (in duplicate) and hybridized with the probe for 16 h, at 42°C, in 5X SSC (0.75M NaCl, 0.075M sodium citrate)/50% formamide, containing 20mM Tris-HCl, pH 7.5, 1X Denhardt's solution, 10% dextran sulfate, and 0.1% SDS. Filters were washed three times, for 10 min each, in 2X SSC/0.1% SDS, at 25 22°C; followed by one wash, for 30 min, in 0.2X SSC/0.1% SDS at 68°C; and exposed to X-ray film (Kodak X-Omat, Eastman Kodak, Rochester, NY) at -70°C. Three overlapping clones were obtained, which together constituted the entire NK-1 receptor gene. The NK-1-encoding fragments were subcloned into pBluescript (Stratagene, La Jolla, CA) and sequenced by the method of double-stranded sequencing (as described above).

30 A full-length human NK-2 receptor cDNA was cloned as follows.

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Oligonucleotide primers corresponding to the putative 3' end and the putative 5' end of the cDNA were designed based on an analysis of the genomic NK-1 sequence and the identification of a putative 5' ATG site. Specifically, these primers corresponded to nucleotides -7 to +11 (i.e., the sense primer) and nucleotides 1190 to 1298 (i.e., the antisense primer) and each included a nested *Bam*HI restriction site at its 5' end. Primers were synthesized as described above. PCR reactions were carried out (also as described above) using about 100 ng of human IM-9 lymphocyte cDNA and 1 μ g of each primer. The PCR amplification involved 5 cycles, each including 1 min. at 95°C, 1.5 min. at 45°C, and 3 min. at 72°C; followed by 25 cycles, each including 1 min. at 95°C, 1.5 min at 55°C, and 3 min. at 72°C; and a final extension of 7 min. at 72°C. Ten percent of the reaction mixture was subjected to secondary PCR, using the same primers and the same cycling conditions as were used for the initial reaction. The material obtained was purified by agarose electrophoresis, digested with *Bam*HI, repurified by agarose electrophoresis, and ligated to *Bam*HI-digested pBluescript SK+ (Stratagene; La Jolla, CA). The PCR product was determined to be a fragment of approximately 1.2 kb. The cDNA clone was sequenced using the methods described above; its nucleic acid sequence and deduced amino acid sequence are shown in FIG. 1 (SEQ ID NO.:3).

By comparison of the NK-1 receptor sequence with other members of the rhodopsin superfamily and by examination of the hydrophobic and hydrophilic regions of the molecule, extracellular and intracellular domains may be deduced. Extracellular domains include: amino acids 1 to 31 (SEQ ID NO.:4); amino acids 90 to 101 (SEQ ID NO.:5); amino acids 170 to 197 (SEQ ID NO.:6); and amino acids 271 to 287 (SEQ ID NO.:7). Intracellular domains

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include: amino acids 60 to 67; amino acids 129 to 148; amino acids 220 to 248; and 310 to 407.

POLYPEPTIDES ACCORDING TO THE INVENTION

- Polypeptides according to the invention include
- 5 the entire human NK-1 receptor as described in FIG. 1 (SEQ. ID. NO.:3). Alternatively, any analog or fragment of the NK-1 receptor capable of interacting with substance P is useful in the invention. Such an interaction may be readily assayed using any of a number
- 10 of standard in vitro methods (see, e.g., Regoli et al., *Tachykinin Antagonists*, eds. Hakanson and Sundler, Elsevier Science Publisher, Amsterdam, 1985). In one particular assay, substance P is adhered to a microtiter plate (using methods similar to those for adhering
- 15 antigens for an ELISA assay) and the ability of labelled NK-1 receptor fragment- or receptor analog- expressing cells (e.g., labelled with ³H-thymidine) to bind the immobilized substance P is used to detect an interaction between substance P and the receptor component.
- 20 Specific receptor analogues of interest include full-length or partial receptor proteins including an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (e.g.,
- 25 valine for glycine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the receptors' ability to interact with substance P (as assayed above).
- 30 Specific receptor fragments of interest include the portions of the receptor deduced to be extracellular. Such regions may be identified by comparison with related proteins of similar structure (e.g., other members of the rhodopsin superfamily); useful regions are those
- 35 exhibiting homology to the extracellular domains of well-

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characterized members of the family. Examples of these regions include, without limitation:

- (a) MDNVLPVDSLSPNISTNTSEPNOQFVQPAWQ (SEQ ID NO.:4; amino acid residues 1 to 31 of Fig. 1; SEQ ID NO.:3);
- 5 (b) FTYAVHNEWYYG (SEQ ID NO.:5; amino acid residues 90 to 101 of Fig. 1; SEQ ID NO.:3);
- (c) TTETMPSRVVCMIEWPEHPNKIYEKVYH (SEQ ID NO.:6; amino acid residues 170 to 197 of Fig. 1; SEQ ID NO.:3); and
- 10 (d) PYINPDLYLKKFIQQVY (SEQ ID NO.:7; amino acid residues 271 to 287 of Fig. 1; SEQ ID NO.:3).

Alternatively, from the primary amino acid sequence, the secondary protein structure and, therefore, the extracellular domain regions may be deduced semi-empirically using a hydrophobicity/hydrophilicity calculation such as the Chou-Fasman method (see, e.g., Chou and Fasman, *Ann. Rev. Biochem.* 47:251, 1978). Hydrophilic domains, particularly ones surrounded by hydrophobic stretches (e.g., transmembrane domains) present themselves as strong candidates for extracellular domains. Finally, extracellular domains may be identified experimentally using standard enzymatic digest analysis, e.g., tryptic digest analysis.

Candidate fragments would be tested for interaction with substance P by the assays described herein (e.g., the assay described above).

POLYPEPTIDE EXPRESSION

Polypeptides according to the invention may be produced by transformation of a suitable host cell with all or part of an NK-1-encoding cDNA or genomic DNA fragment (e.g., described above) in a suitable expression vehicle, and expression of the receptor.

Those skilled in the field will understand that any of a wide variety of expression systems may be used to provide the recombinant receptor protein. The precise

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host cell used is not critical to the invention, however the following host cells are preferred: Chinese Hamster Ovary (CHO) cells, Madin-Darby Canine Kidney (MDCK) cells, COS cells, and fibroblast cells, such as mouse 3T3
5 cells. Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; Accession Nos. CCL 61, CCL 34, CRL 1650, and CCL 163, respectively). The method of transfection and the choice of expression vehicle will depend on the
10 host system selected. Mammalian cell transfection methods are described, e.g., in Ausubel et al. (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1989); expression vehicles may be chosen from those provided, e.g., in *Cloning Vectors: A Laboratory Manual*
15 (P.H. Pouwels et al., 1985, Supp. 1987).

One particularly preferred expression system is the mouse 3T3 fibroblast host cell transfected with a pMAMneo expression vector (Clontech, Palo Alto, CA). pMAMneo provides: an RSV-LTR enhancer linked to a
20 dexamethasone-inducible MMTV-LTR promoter, an SV40 origin of replication (which allows replication in mammalian systems), a selectable neomycin gene, and SV40 splicing and polyadenylation sites. DNA encoding the NK-1 receptor or an appropriate receptor fragment or analog
25 (as described above) would be inserted into the pMANeo vector in an orientation designed to allow expression. The recombinant receptor protein would be isolated as described below. Other preferable host cells which may be used in conjunction with the pMAMneo expression
30 vehicle include COS cells and CHO cells (ATCC Accession Nos. CRL 1650 and CCL 61, respectively).

In another preferred expression system, the NK-1 receptor protein (or receptor fragment or analog) is produced by a stably-transfected mammalian cell line. A
35 number of vectors suitable for stable transfection of

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mammalian cells are available to the public, e.g., see Pouwels et al. (supra); methods for constructing such cell lines are also publically available, e.g., in Ausubel et al. (supra). In one example, cDNA encoding
5 the receptor (or receptor fragment or analog) is cloned into an expression vector which includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the NK-1 receptor-encoding gene into the host cell chromosome is selected for by
10 inclusion of 0.01-300 μ M methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types. Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene.
15 Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this
20 purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR⁻ cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of
25 a stably-transfected cell line or DHFR-mediated gene amplification.

Yeast cells may also be used as a host system. Yeast vectors into which the NK-1 receptor- or receptor fragment- or analog-encoding DNA may be cloned are
30 publically available, and many are described in Pouwels et al. (supra). Methods of yeast transformation are described in Ausubel et al. (supra).

Once the recombinant NK-1 receptor protein (or fragment or analog, thereof) is expressed, it is
35 isolated, e.g., using affinity chromatography. In one

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xample, substance P or anti-NK-1 receptor antibodies (described below) may be attached to a column and used to isolate intact receptor or receptor fragments or analogues. Lysis and fractionation of receptor-harboring
5 cells prior to affinity chromatography may be performed by standard methods (see, e.g., Ausubel et al., supra). Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography.

10 Receptors of the invention, particularly short receptor fragments, can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984, The Pierce Chemical Co., Rockford, IL).

15 SCREENING FOR NK-1 RECEPTOR ANTAGONISTS

As discussed above, one aspect of the invention features screening for compounds that antagonize the interaction between substance P and the NK-1 receptor, thereby preventing or reducing the cascade of events that
20 are mediated by that interaction. The elements of the screen are the substance P neurotransmitter (or a suitable receptor-binding fragment or analog thereof) and recombinant NK-1 receptor (or a suitable receptor fragment or analog, as outlined above) configured to
25 permit detection of binding.

Substance P may be obtained from the Sigma Chemical Co. (St. Louis, MO). Alternatively, it may be produced by standard methods of chemical synthesis or recombinant DNA technology given its known amino acid
30 sequence: Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂ (SEQ ID NO.:2).

Preferably, the NK-1 receptor component is produced by a cell that naturally presents substantially no receptor, e.g., by engineering such a cell to contain
35 nucleic acid encoding the receptor component in an

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appropriate expression system. Suitable cells are, e.g., those discussed above with respect to the production of recombinant receptor, most preferably mouse 3T3 fibroblast cells.

5 The assay is preferably performed by fixing the cell expressing the NK-1 receptor component to a solid substrate (e.g., a test tube or microtiter well) by means well known to those in the art, and presenting labelled substance P or a fragment or analog thereof to
10 the cell in the presence of the candidate antagonist. Binding is assayed by the detection label in association with the receptor component (and, therefore, in association with the solid substrate). Molecules which specifically interfere with labelled substance P binding
15 are considered to be useful in the invention.

 The assay format may be any of a number of suitable formats for detecting specific binding, such as a radioimmunoassay format. Preferably, cells transiently or stably transfected with an NK-1 receptor expression
20 vector are immobilized on a microtiter plate and reacted with substance P (or an active fragment or analog thereof) which is detectably labelled, e.g., with a radiolabel or an enzyme which can be assayed, e.g., alkaline phosphatase or horseradish peroxidase.

25 Alternatively, substance P may be adhered to the microtiter plate (using methods similar to those for adhering antigens for an ELISA assay) and the ability of labelled NK-1 receptor expressing cells (e.g., labelled with ³H-thymidine) can be used to detect specific
30 receptor binding to the immobilized substance P.

 In one particular example, a vector expressing the NK-1 receptor (or receptor fragment or analog) is transfected into COS-7 cells (ATCC Accession No. CRL 1651) by the DEAE dextran-chloroquine method (Ausubel et
35 al., supra). Expression of the receptor protein confers

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binding of the substance P neurotransmitter to the cells; substance P does not bind to untransfected host cells or cells bearing the parent vector alone. 10 cm. tissue culture dishes are seeded with freshly trypsinized NK-1
5 receptor-expressing COS-7 cells (750,000 cells, dish) 12-18h post-transfection. Forty-eight hours later, triplicate dishes are incubated with 2nM radioiodinated substance P (~300 cpm/fmol). This is used as a "control" against which antagonist assays are measured. Such
10 antagonist assays involve incubation of the NK-1 receptor-expressing cells with an equivalent amount of labelled substance P in combination with an appropriate amount of candidate antagonist. An antagonist useful in the invention blocks labelled substance P binding to the
15 immobilized receptor-expressing cells.

Appropriate candidate antagonists include NK-1 receptor fragments, particularly fragments containing an extracellular domain (described above); such fragments would preferably including five or more amino acids.
20 Other candidate antagonists include non-peptide compounds designed or derived from analysis of the receptor, as well as anti-NK-1 receptor antibodies.

ANTI-NK-1 RECEPTOR ANTIBODIES

NK-1 receptor or receptor fragments or analogues
25 (described above) may be used to raise antibodies by any of the conventional methods well known to those skilled in the art. For example, the cDNA sequence of the NK-1 receptor can be used to select short peptide sequences which can be synthesized (e.g., by chemical synthesis or
30 recombinant DNA techniques) and used to immunize animals, e.g., rabbits, in order to generate antibodies. The antibodies may be polyclonal or monoclonal. Polyclonal antibodies may be enriched in anti-receptor activity, e.g., by column purification (i.e., by using receptor or
35 receptor fragments or analogues immobilized on a column

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to screen out the desired antibody, see, e.g., Ausubel et al., supra). Desired monoclonal antibody-producing hybridomas may also be selected by stimulating and then screening with receptor or receptor fragments or analogues, using standard immunological techniques (see, e.g., Ausubel et al., supra).

THERAPEUTICS AND VACCINES

Particularly suitable therapeutics are the antagonistic receptor fragments (described above) formulated in an appropriate buffer such as physiological saline. Where it is particularly desirable to mimic the receptor conformation at the membrane interface, the fragment may include transmembrane residues adjacent to the extracellular domain of the receptor. In this case, the fragment may be associated with an appropriate lipid fraction (e.g., in lipid vesicles or attached to fragments obtained by disrupting a cell membrane). Alternatively, anti-NK-1 receptor antibodies produced as described above may be used as a therapeutic. Again, the antibodies would be administered in a pharmaceutically-acceptable buffer (e.g., physiological saline). If appropriate, the antibody preparation may be combined with a suitable adjuvant.

The therapeutic preparation is administered in accordance with the condition to be treated. Ordinarily, it will be administered intravenously, at a dosage that provides suitable competition for substance P binding. Alternatively, it may be convenient to administer the therapeutic orally, nasally, or topically, e.g., as a liquid or a spray, respectively. Again, the dosage would be adjusted to provide suitable competition for NK-1 binding. Treatment may be repeated as necessary for alleviation of pain or disease symptoms.

Because the NK-1 receptor is involved in the transmission of stimuli from the peripheral nervous

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system, the therapeutic peptides may be useful for alleviation of pain and may be particularly useful when administered to reduce chronic pain. This association with the nervous system also suggests that the NK-1
5 receptor may facilitate the development of a therapeutic to treat mental-health disorders, such as schizophrenia, or reduce the symptoms of anxiety or stress. Further, the NK-1 receptor's involvement in mononuclear and polymorphonuclear leukocyte activation suggests that an
10 NK-1 receptor antagonist can be used to treat leukocyte-proliferative diseases, e.g., inflammatory diseases, such as arthritis and asthma.

The antibodies of the invention, in a suitable buffer and, if appropriate, including an adjuvant, may
15 also be used as a protective vaccine. Such a vaccine would be administered in a dosage that provides suitable competition for substance P binding over the long-term. Such a vaccine would be useful, e.g., to individuals suffering from chronic pain or any of the disorders
20 (e.g., inflammatory diseases, mental-health disorders) described above.

Other embodiments are within the following claims.

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Claims

1. Human recombinant NK-1 receptor or a fragment thereof.
2. The receptor of claim 1 comprising an amino
5 acid sequence substantially identical to the amino acid sequence shown in Fig. 1 (SEQ ID NO.:3).
3. A substantially isolated polypeptide which is a fragment of a human NK-1 receptor comprising an extracellular domain capable of binding substance P
10 neurotransmitter.
4. A polypeptide selected from the group comprising:
 - (a) MDNVLPVDSLSPNISTNTSEPNQFVQPAWQ (SEQ ID NO.:4; amino acid residues 1 to 31 of Fig. 1; SEQ ID NO.:3);
 - 15 (b) FTYAVHNEWYYG (SEQ ID NO.:5; amino acid residues 90 to 101 of Fig. 1; SEQ ID NO.:3);
 - (c) TTETMPSRVVCMIIEWPEHPNKIYEKVYH (SEQ ID NO.:6; amino acid residues 170 to 197 of Fig. 1; SEQ ID NO.:3);
 - (d) PYINPDLYLKKFIQQVY (SEQ ID NO.:7; amino acid
20 residues 271 to 287 of Fig. 1; SEQ ID NO.:3); and
 - (e) fragments or analogues of (a)-(d) which are capable of binding human NK-1 neurotransmitter.
5. The polypeptide of claim 4 further characterized in that said polypeptide is a recombinant
25 polypeptide.
6. Purified DNA which encodes a receptor or receptor fragment of claim 1 or a polypeptide of claims 2, 3, or 4.

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7. A vector containing the DNA of claim 6, said vector being capable of directing expression of the protein encoded by said DNA in a vector-containing cell.

8. A cell which contains the vector of claim 7.

5 9. The cell of claim 8, said cell being a eukaryotic cell.

10. The cell of claim 9, said cell being a mammalian cell.

11. A method of producing recombinant NK-1
10 receptor protein or a fragment thereof comprising,
 providing a cell transformed with DNA encoding the
 NK-1 receptor of a fragment thereof positioned for
 expression in said cell;
 culturing said transformed cell under conditions
15 for expressing said DNA; and
 isolating said recombinant human NK-1 receptor
 protein.

12. A purified antibody which binds
 preferentially to a receptor or fragment of claim 1 or a
20 polypeptide of claims 2 or 3.

13. The antibody of claim 12, wherein said
 antibody neutralizes the biological activity in vivo of a
 receptor or fragment of claim 1 or a polypeptide of
 claims 2 or 3.

25 14. A therapeutic composition comprising as an
 active ingredient recombinant receptor according to claim
 1, a polypeptide according to claims 2 or 3, or an
 antibody according to claims 12 or 13, said active

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ingredient being formulated in a physiologically-acceptable carrier.

15. The therapeutic composition of claim 14 wherein said active ingredient comprises the recombinant
5 NK-1 receptor of claim 1 or a polypeptide of claims 2 or 3 anchored within the membrane of a cell.

16. A method of reducing pain in a mammal comprising administering the therapeutic composition of claims 14 or 15 to said mammal in a dosage effective to
10 antagonize an interaction between substance P neurotransmitter and an NK-1 receptor.

17. A method of treating an inflammatory disease in a mammal comprising administering the therapeutic composition of claims 14 or 15 to said mammal in a dosage
15 effective to antagonize an interaction between substance P neurotransmitter and an NK-1 receptor.

18. The method of claim 17, wherein said inflammatory disease is arthritis.

19. The method of claim 17, wherein said
20 inflammatory disease is asthma.

20. A method of treating a mental illness in a mammal comprising administering the therapeutic composition of claims 14 or 15 to said mammal in a dosage effective to antagonize an interaction between substance
25 P neurotransmitter and an NK-1 receptor.

21. The method of claim 20, wherein said mental illness is schizophrenia.

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22. A method of treating a physiological response to stress in a mammal comprising administering the therapeutic composition of claims 14 or 15 to said mammal in a dosage effective to antagonize an interaction
5 between substance P neurotransmitter and an NK-1 receptor.

23. A method of screening candidate compounds for the ability to antagonize interaction between substance P neurotransmitter and an NK-1 receptor, said method
10 comprising:

a) mixing a candidate antagonist compound with a first compound comprising the recombinant NK-1 receptor or fragment of claim 1 or a polypeptide of claims 2 or 3, or an antibody of claims 12 or 13 on the one hand and
15 with a second compound comprising substance P neurotransmitter;

b) determining whether said first and second compounds bind; and

c) identifying antagonistic compounds as those
20 which interfere with the binding of the first compound to the second compound.

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ATGGATAACGTCTCCCGGTGGACTCAGACCTCTCCCCAACATCTCCACTAACACCTCG
M D N V L P V D S D L S P N I S T N T S
GAACCCAATCAGTTCGTGCAACCAGCCTGGCAAATTGTCTTTGGGCAGCTGCTTACAG
E P N Q F V Q P A W Q I V L W A A A Y T
GTCATTGTGGTGACCTCTGTGGTGGGCAACGTGGTAGTGATGTGGATCATCTTAGCCAC
V I V V T S V V G N V V V M W I I L A H
AAAAGAATGAGGACAGTGACGAACCTATTTTCTGGTGAACCTGGCCTTCGCGGAGGCCTCC
K R M R T V T N Y F L V N L A F A E A S
ATGGCTGCATTCAATACAGTGGTGAACCTCACCTATGCTGTCCACAACGAATGGTACTAC
M A A F N T V V N F T Y A V H N E W Y Y
GGCCTGTTCTACTGCAAGTTCCACAACCTTCTTCCCCATCGCCGCTTGCTTCGCCAGTATC
G L F Y C K F H N F F P I A A C F A S I
TACTCCATGACGGCTGTGGCCTTTGATAGGTACATGGCCATCATACATCCCCTCCAGCCC
Y S M T A V A F D R Y M A I I H P L Q P
CGGCTGTGAGCCACAGCCACCAAAGTGGTCATCTGTGTCATCTGGGTCTGGCTCTCCTG
R L S A T A T K V V I C V I W V L A L L
CTGGCCTTCCCCAGGGCTACTACTCAACCACAGAGACCATGCCAGCAGAGTCGTGTGC
L A F P Q G Y Y S T T E T M P S R V V C
ATGATCGAATGGCCAGAGCATCCGAACAAGATTTATGAGAAAGTGACCACATCTGTGTG
M I E W P E H P N K I Y E K V Y H I C V
ACTGTGCTGATCTACTTCTCCCCCTGCTGGTGAATTGGCTATGCATACCATAGTGGGA
T V L I Y F L P L L V I G Y A Y T I V G
ATCACACTATGGGCCAGTGAGATCCCCGGGGACTCCTCTGACCGCTACCACGAGCAAGTC
I T L W A S E I P G D S S D R Y H E V Q
TCTGCCAAGCGCAAGGTGGTCAAATGATGATTGTCGTGGTGTGCACCTTCGCCATCTGC
S A K R K V V K M M I V V V C T F A I C
TGGCTGCCCTTCACATCTTCTTCTCCTGCTGCTACATCAACCCAGATCTCTACCTGAAG
W L P F H I F F L L P Y I N P D L Y L K
AAGTTTATCCAGCAGGTCTACCTGGCCATCATGTGGCTGGCCATGAGCTCCACCATGTAC
K F I Q Q V Y L A I M W L A M S S T M Y
AACCCCATCATCTACTGCTGCCTCAATGACAGGTTCCGTCTGGGCTTCAAGCATGCCTTC
N P I I Y C C L N D R F R L G F K E A F
CGGTGCTGCCCTTCATCAGCGCCGGCGACTATGAGGGGCTGGAATGAAATCCACCCGG
R C C P F I S A G D Y E G L E M K S T R
TATCTCCAGACCCAGGGCAGTGTGTACAAAGTCAGCCGCTGGAGACCACCATCTCCACA
Y L Q T Q G S V Y K V S R L E T T I S T
GTGGTGGGGGGCCACGAGGAGGAGCCAGAGGACGGCCCAAGGCCACACCCTCGTCCCTG
V V G A H E E E P E D G P K A T P S S L
GACCTGACCTCCAACCTGCTCTTACGAAGTGACTCCAAGACCATGACAGAGAGCTTCAGC
D L T S N C S S R S D S K T M T E S F S
TTCTCCTCCAATGTGCTCTCCTAGGGATCC

FIG. 1

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02007

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC (5): C07K 3/00, 7/00, 7/08, 13/00 US CL : 514/2; 530/350, 300; 435/69.1		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	514/2; 530/350, 300; 435/69.1	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
APS, DIALOGUE, REGISTRY, INTELLIGENETICS. SEARCH TERMS: SEQUENCES PROVIDED, NK-1 RECEPTOR, RECOMBINANT, ISOLAT?, IDENTIF?, SEQUENCE? ?.		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, VOLUME 264 NUMBER 30, ISSUED 25 OCTOBER 1989, (USA), YOKOTA ET AL, "MOLECULAR CHARACTERIZATION OF A FUNCTIONAL CDNA FOR RAT SUBSTANCE P RECEPTOR", PAGES 17649-17652.	1-5, 11 AND 16
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, VOLUME 266 NUMBER 7, ISSUED 05 MARCH 1991, HERSHEY ET AL, "ORGANIZATION, STRUCTURE, AND EXPRESSION OF THE GENE ENCODING THE RAT SUBSTANCE P RECEPTOR", PAGES 4366-4374.	1-5, 11 AND 16
X,P	BIOCHEMISTRY, VOLUME 30, NUMBER 44, ISSUED 05 NOVEMBER 1991, GERARD ET AL, "HUMAN SUBSTANCE P RECEPTOR (NK-1): ORGANIZATION OF THE GENE CHROMOSOMAL LOCALIZATION, AND FUNCTIONAL EXPRESSION OF CDNA CLONES", PAGES 10640-10646.	1-5, 11 AND 16
X,P	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, VOLUME 179, NUMBER 3, ISSUED 30 SEPTEMBER 1991, TAKEDA ET AL, "MOLECULAR CLONING, STRUCTURAL CHARACTERIZATION AND FUNCTIONAL EXPRESSION OF THE HUMAN SUBSTANCE P RECEPTOR", PAGES 1232-1240.	1-5, 11 AND 16
<p>* Special categories of cited documents:¹⁶</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²		Date of Mailing of this International Search Report ²
03 June 1992		19 JUN 1992
International Searching Authority ¹		Signature of Authorized Officer ²⁰
ISA/US		<i>Christopher J. Dubrule</i> CHRISTOPHER J. DUBRULE

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FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X, P	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, VOLUME 180, NUMBER 2, ISSUED 31 OCTOBER 1991, HOPKINS ET AL. "ISOLATION AND CHARACTERISATION OF THE HUMAN LUNG NK-1 RECEPTOR CDNA", PAGES 1110-1117.	1-5, 11 AND 16
X, P	JO, A. 3-133998 (MITSUBISHI KASEI CORP.) 07 JUNE 1991.	1-5, 11 AND 16
Y	SCIENCE, VOLUME 247, ISSUED 23 FEBRUARY 1990, HERSHEY ET AL. "MOLECULAR CHARACTERIZATION OF A FUNCTIONAL CDNA ENCODING THE RAT SUBSTANCE P RECEPTOR", PAGES 958-962.	1-5, 11 AND 16

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

1. ☐ Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. ☐ Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

Please See Attached Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-5, 11 AND 16 (Telephone Practice)

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PREVIOUS SHEETS

VI. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. CLAIMS 1-5, 11 AND 16, DRAWN TO THE NK-1 RECEPTOR AND FRAGMENTS THEREOF, A METHOD OF MAKING THE RECEPTOR UTILIZING A TRANSFORMANT, AND A METHOD OF REDUCING PAIN BY ADMINISTERING THE RECEPTOR OR FRAGMENT THEREOF. CLASSIFIED IN CLASS 530, SUBCLASS 350.

II. CLAIM 6, DRAWN TO DNA ENCODING NK-1 RECEPTOR AND FRAGMENTS THEREOF. CLASSIFIED IN CLASS 536, SUBCLASS 27.

III. CLAIM 7, DRAWN TO AN EXPRESSION VECTOR. CLASSIFIED IN CLASS 935, SUBCLASS 22.

IV. CLAIMS 8-10, DRAWN TO TRANSFORMED CELLS. CLASSIFIED IN CLASS 935, SUBCLASS 66.

V. CLAIMS 12-13, DRAWN TO ANTIBODIES TO THE RECEPTOR. CLASSIFIED IN CLASS 530, SUBCLASS 387.

VI. CLAIMS 14-15, DRAWN TO THERAPEUTIC COMPOSITIONS CONTAINING THE RECEPTOR OR ANTIBODIES TO THE RECEPTOR. CLASSIFIED IN CLASS 514, SUBCLASS 2.

VII. CLAIMS 17-19, DRAWN TO A METHOD OF TREATING INFLAMMATORY DISEASE. CLASSIFIED IN CLASS 514, SUBCLASS 2.

VIII. CLAIMS 20-21, DRAWN TO METHODS OF TREATING MENTAL ILLNESS. CLASSIFIED IN CLASS 514, SUBCLASS 2.

IX. CLAIM 22, DRAWN TO A METHOD OF TREATING STRESS. CLASSIFIED IN CLASS 514, SUBCLASS 2.

X. CLAIM 23, DRAWN TO A METHOD OF SCREENING CANDIDATE COMPOUNDS. CLASSIFIED IN CLASS 436, SUBCLASS 501.

GROUP I REPRESENTS A PRODUCT, ITS METHOD OF MAKING, AND A FIRST METHOD OF USE. GROUPS II-VI REPRESENT PRODUCTS WHICH ARE DISTINCT FROM THAT OF GROUP I AND FROM EACH OTHER AS EVIDENCED BY THEIR DIFFERENT CLASSIFICATIONS. GROUPS VII-X REPRESENT ALTERNATIVE METHODS OF USE OF THE PRODUCT. THESE METHODS ARE DISTINCT FROM EACH OTHER BECAUSE OF THEIR RECOGNIZED DIVERGENT SUBJECT MATTER, I.E. TREATING INFLAMMATORY DISEASE VS. TREATING STRESS. PCT RULES 13.1 AND 13.2 DO NOT PROVIDE FOR MORE THAN ONE METHOD OF MAKING, ONE PRODUCT MADE, AND ONE METHOD OF USE.